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Biofilms in water, its role and impact in human disease transmission

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Understanding the mechanism of biofilm formation is the first step in determining its function and, thereby, its impact and role in the environment. Extensive studies accomplished during the past few years have elucidated the genetics and biochemistry of biofilm formation. Cell-to-cell communication, that is, quorum sensing, is a key factor in the initiation of biofilm. Occurrence of viable but nonculturable bacteria, including *Vibrio cholerae* in biofilms has been reported and most likely such cells were overlooked previously because appropriate methods of detection were not employed. For this reason discovery and investigation of this important bacterial ecological niche in the environment were impeded.

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Introduction

A biofilm has been defined as an assemblage of microbial cells that is surrounded by a matrix of exopolysaccharide (EPS) secreted by those cells. Biofilms can be composed of a pure culture, but more commonly comprise a community of mixed microbial species. The formation of a biofilm is a developmental process in which a quorum sensing signal molecule, an auto-inducer, functions to induce the secretion of EPS and leads to the formation of a characteristic three-dimensional biofilm architecture [1••]. Biofilm formation can be viewed as a survival mechanism for bacteria; bio film can provide protection from toxic compounds, such as antibiotics, thermal stress, and predation [2]. Biofilms in drinking water containing *Escherichia coli*, *Aeromonas*, and *Pseudomonas* spp., harbor potential risk to human health if pathogenic forms are present [3]. The number of cells in a biofilm may reach as high as 1.0×10^9 cells per clump which, in most cases, can comprise an infectious dose of a pathogen. However, survivability of bacterial cells and retention of their potential to cause disease when present in a biofilm remains to be fully elucidated [4••].

Formation of biofilm in aquatic environments

Bacteria in biofilms in an aquatic environment are rarely planktonic, instead are associated with surfaces that can include living tissues, indwelling medical devices, or industrial or potable water system piping, attachment helping the bacteria from being swept away [2]. Production of alginate, an exopolysaccharide present as an extracellular matrix in *Pseudomonas aeruginosa* biofilm, is induced upon the contact of the cells with a surface. Laboratory microcosm experiments suggest cells of *V. cholerae* form biofilms on biotic and abiotic surfaces, thereby protecting themselves with this exopolymer barrier [5].

Other factors, such as less soluble or metabolizable large organic compounds, including humic acids, that are adsorbed onto aquatic surfaces, and chitinous exoskeletons of crustaceans provide nutrition directly to the attached bacteria. Ca^{2+}, which is abundant in marine and brackish water ecosystems, plays an important role in the formation of biofilms by directly stabilizing intercellular interactions, as has been shown for *P. aeruginosa* and *Streptococcus downei*, presumably by forming intercellular salt bridges. Biofilms in seawater similarly can involve chromium, nickel, and molybdenum, used as alloying elements to resist corrosion of stainless steel bodies of marine vessels. Biofilms on those surfaces, however, do become corroded over time [6].

Interestingly, the correlation between sea surface height in the Bay of Bengal and cholera epidemics in Bangladesh, can be explained by seawater, carrying particulates and plankton colonized with cholera bacteria in the form of biofilms inundating adjacent estuaries, and ultimately introducing the cholera bacteria to brackish

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14. ABSTRACT

Understanding the mechanism of biofilm formation is the first step before determining its function and, thereby, the impact and role in the environment. Cell to cell communication, i.e., quorum sensing, is a key factor in the initiation of biofilm, yet extensive studies accomplished during the past few years have elucidated the genetics and biochemistry of biofilm formation. Occurrence of viable but nonculturable bacteria, including Vibrio cholerae in biofilms has been reported and most likely such cells were overlooked previously because appropriate methods of detection were not employed. In fact, for this reason discovery and investigation of this important bacterial ecological niche in the environment were impeded.

15. SUBJECT TERMS

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ecosystems [4••,7]. *V. cholerae* serogroups O1 and O139 have been shown to survive year round, mostly in a nonculturable state, within clusters of biofilm in the ecosystem adjacent to the coast of the Bay of Bengal, accounting for the endemic cholera in that geographic region [7]. Biofilm-like, multicellular clumps of *V. cholerae* are found in human stools [8], and a recent epidemiological and ecological study offered firm evidence that bodies of water serving as drinking water sources harbored biofilm-bound, nonculturable *V. cholerae* O1 cells between epidemics occurring in the coastal villages of the Bay of Bengal seasonally [9]. Subsequent studies also showed that nonculturable *V. cholerae* O1 cells naturally present in biofilms were more adapted to the aquatic ecosystem, compared to *V. cholerae* shed in cholera stools [4••], and discharged into the aquatic ecosystem. The latter were found to remain infective for a relatively shorter period. *V. cholerae* cells within naturally occurring biofilms in the aquatic ecosystem, a significant role in the ecology and seasonal epidemics of cholera in Bangladesh.

Analysis of biofilms and detection of the organisms

Microscopy continues to be the most widely used tool in the analysis of biofilms. Confocal scanning laser microscopy (CSLM) captures top-down images of mature biofilms that can be viewed in a qualitative manner. Additionally, those images can be sectioned along the xz axis and reconstructed to give sagittal or orthogonal profiles, for which the depth, and subsequently volume, of the biofilm can be quantified [10–12]. There are a number of software packages, such as COMSTAT or IMARIS, that can assist in this type of analysis [10,13]. Phase contrast light microscopy and epifluorescence microscopy are used primarily to examine early development of biofilms, that is cell attachment and migration to form microcolonies, and in those cases where CSLM is not available [4••]. Occasionally, scanning and transmission electron microscopy are used.

Nonmicroscopic methods for the analysis of biofilm biomass include staining with crystal violet (absorbance at 570 nm), Syto9, fluorescein diacetate (FDA), resazurin, tetrazolium salts (CTC, XTT), and dimethyl methylene blue (DMMB) [4••,11,12,14•,15•]. These assays principally work the same, except they stain in different ways; e.g., cells and biofilm matrix, viable cells, and/or the polysaccharide matrix. Attached cells are incubated with the staining agent, rinsed to remove unattached cells, and then quantified, based on fluorescence absorbance. For bacterial enumeration, the biofilm is first mechanically disrupted (glass beads), serially diluted and colony-forming units determined by plate count [12].

Culture conditions

Flow cell biofilm reactors, derivatives of chemostats, have been used primarily for studying biofilms. Most are driven by a peristaltic pump and include single pass systems and recycling reactors [13]. The biofilm bait can be a silicone tubing, such as is used in catheters, or glass capillary [10,13]. Many less expensive alternatives to flow cells have been described. In one study, researchers used a chambered cover glass system (Nunc Lab-Tek), originally designed for tissue culture [10]. Borosilicate slide cover glasses (22 mm × 22 mm) submerged in media in 50 ml conical centrifuge tubes were employed with good results by Liu *et al.* [12]. For all culture methods, the resultant biofilm can be assayed by microscopy. When nonmicroscopic methods are used, the biofilm can be cultured in polyvinyl chloride or polystyrene microtiter plates or borosilicate glass tubes [11,14•].

Role of biofilms in causing disease and disease transmission

It has been long known that bacteria can adhere to solid surfaces and form a slimy, slippery coat (i.e. biofilm). Furthermore, it has been suggested that biofilms play a significant role in the transmission and persistence of human disease. For human pathogenic bacteria, biofilms offer protection to the bacteria from the host immune system and allow those bacteria to withstand killing doses of antibiotics. For example, in the medical community, one of the greatest concerns has been microbial biofilms on indwelling medical devices (e.g. prosthetic heart valves) or other devices used in the healthcare environment that can harbor biofilms. In humans, *V. cholerae* biofilms in the gut of cholera patients may be protected from acid pH and/or antibiotic, thereby serving as a vehicle for fecal-oral mode of transmission of the disease. In the natural environment, biofilms can also provide a safe haven for pathogenic bacteria, protecting them from a variety of physiochemical stresses, including UV light, oxidative stress, dehydration, and biocidal agents. Additionally, since bacteria in the natural environment are subject to predation by protozoa, bacterivorous microorganisms (e.g. *Bdellovibrio* spp.), and bacteriophages, it is probable that biofilms provide a mechanism for the persistence of bacterial-forming bacterial pathogens in the environment.

There is an increasing realization of the importance of the world's oceans as a source of potentially pathogenic microorganisms. Human bacterial pathogens associated with the marine environment and the diseases they cause have been reviewed [16]. Several bacterial pathogens found in the marine environment produce biofilms. While progress is being made in elucidating molecular and genetic mechanisms by which these bacteria produce biofilms, its role in disease transmission, by far the most detailed work has been done on *V. cholerae*, the causative agent of cholera.

Biofilm formation in *V. cholerae* is a multistep developmental process controlled by several regulatory path-

ways. In particular, recent evidence suggests that two main systems are responsible for biofilm formation and regulation in *V. cholerae*, namely, the matrix exopolysaccharide and quorum sensing. *V. cholerae* has been shown to undergo phenotypic (phase) variation, generating two morphologically distinct variants, smooth and rugose. This phase variation has been implicated in its adaption to variation in its natural aquatic ecosystem. In particular, the rugose variant has an enhanced capacity to form biofilms [17], a phenotype mediated, in part, by increased production of exopolysaccharide VPS (*Vibrio* polysaccharide synthesis). New data suggest that the gene, *rbm*A (rugosity and biofilm structure modulator A), is required for rugose colony formation and integrity of the biofilm structure in *V. cholerae* [17]. Bacteria can engage in social behavior in which they coordinate expression of certain genes or phenotypes using quorum-sensing regulation. Essentially, individual cells detect the density of cells around them and activate expression of target genes when the density is high. It has been established that *V. cholerae* employs cell-to-cell communication, namely quorum sensing, to control biofilm formation. Specifically, quorum sensing and 3′,5′-cyclic diguanylic acid (c-di-GMP) act reciprocally to control biofilm formation. In *V. cholerae*, quorum sensing represses biofilm formation and c-di-GMP, an intracellular second messenger, activates biofilm formation. Recent genetic evidence shows that a protein, HapR, which is produced at high cell density represses biofilm formation by two distinct mechanisms [18••]. First, HapR controls transcription of 14 genes encoding a group of proteins that synthesize and degrade c-di-GMP, resulting in a net reduction in cellular c-di-GMP levels at high cell density and, thereby, a decrease in biofilm production. Second, HapR binds directly to and represses the expression of the biofilm transcriptional activator, *vpsT*, also leading to decrease in biofilm formation.

Biofilm formation by pathogens in the environment can play a role in the transmission of those pathogens. For example, an obvious mechanism by which microbial pathogens active in biofilms cause disease is by the seeding and dispersal of a large number of cells that subsequently initiate an infection. This phenomenon was shown to be the case for *V. cholerae* in surface waters in a cholera-endemic area in Bangladesh, where large numbers of toxigenic *V. cholerae* in the viable but nonculturable state were detected in biofilms. Since it had been shown that cholera patients shed high concentrations of *V. cholerae in vivo*-formed biofilms, shedding clumps of cells appears to be an efficient way of delivering an infectious dose of organisms [8]. Thus, biofilm formation by *V. cholerae* can play a major role in its ecology and in the epidemiology of cholera and, very likely, other water-borne bacterial pathogens can do so as well.

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- •• of outstanding interest

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